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Research Article

Prevalence and Antimicrobial Resistance of *Campylobacter* spp. in the Raw Milk of Backyard-Raised Carabaos (*Bubalus bubalis*) in the Philippines

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Abstract

Background and Objective: The World Health Organization listed *Campylobacter* spp. as one of the most common food-borne bacterial pathogens worldwide. In the Philippines, *Campylobacter* contamination in chicken are well established but there is a research gap on the presence and antimicrobial resistance of *Campylobacter* spp., in raw carabaos' milk. This study aims to determine the prevalence of *Campylobacter* spp., in the raw milk and its resistance to common antimicrobial agents. **Materials and Methods:** This study utilized the combination of conventional culture method and a commercial milk bacterial DNA isolation kit to detect the presence of *Campylobacter coli* and *Campylobacter jejuni* in raw milk. In both methods, *Campylobacters* were genotyped with primers that encode for lipid A; while antibiotic resistance was determined using primers for tetracycline (*tetO*) and ampicillin (*blaOXA-61*) resistance genes. **Results:** Out of 107 raw milk samples, *C. coli* was detected in 0.94% of the samples using conventional culture method and on 6.54% (95% CI, 3.2-12.9%) using the commercial kit. No *C. jejuni* were detected using both methods. No genes that encode for tetracycline and ampicillin resistance were detected but phenotypic testing showed intermediate resistance to ampicillin. During the analysis, several *Campylobacter*-like colonies grew on the selective media but 16S gene sequencing revealed the colonies to be *Acinetobacter baumannii* (59%) and *Pseudomonas aeruginosa* (23%). **Conclusion:** Results confirmed the presence of *C. coli* in raw carabaos milk which possess resistance against ampicillin, suggesting that a review of the milk handling protocol in backyard farms is necessary. Further, the difficulty encountered in the isolation of *Campylobacters* can be a source of bias and must be considered in future surveillance programs for this food-borne pathogen.

Key words: *lpxA* genes, mCCDA, 16S sequencing, *Acinetobacter baumannii*, competing microflora, ampicillin

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Milk can be regarded as a complete food because it provides the major macronutrients and the necessary vitamins and minerals for human growth and well-being. In the Philippines majority of milk consumed were imported from overseas such as New Zealand, USA and Australia with milk imports from the US increasing by 45%[1]. The Philippine carabao, therefore, remains a readily accessible and inexpensive alternative source of milk and milk products for the country. However, contamination of milk by microorganisms can occur[2] and while the nutrient-rich milk provides humans with needed nutrients, it can also become a growth medium for microorganisms creating public health safety concerns[3]. The problem of contamination can be significant in backyard-raised dairy carabaos, where the protocol for proper cleaning and disinfection of the animal and milking equipment may not be followed or the standard milk processing protocol, such as pasteurization, is not done.

Salmonella enterica, *Escherichia coli* and *Campylobacter* spp., are the three most common bacterial contaminants of milk[4] and the leading causes of diarrheal diseases in the world[5]. Campylobacteriosis is particularly important because it is associated post infection with Guillain Barre syndrome, a neurological disorder[6] and Reiter's Syndrome, a form of reactive arthritis[7,8]. The problem with campylobacteriosis also extends to the socio-economic effects of the disease i.e., healthcare and non-healthcare costs, loss of productivity, etc.[9]. Identifying the possible sources of *Campylobacter* spp., contamination to prevent these problems is therefore a priority.

Worldwide, *Campylobacter* spp., was detected from a variety of foodstuffs including chicken, pork and beef and milk and milk products[10,11]. In the Philippines, studies on *Campylobacter* prevalence in chicken[12,13], dog faeces[14] and cloacal swabs in bats[15] have been conducted but there is no data yet on the presence and antimicrobial resistance of *Campylobacter* spp., in raw carabaos' milk. Studies on the presence of *Campylobacter* in food, animals and the environment in the country should be a priority because *Campylobacter* spp., has been found in ~3% of 2,908 hospital patients with or without symptoms of diarrhea[16] and in 4 of 7 (57%) soldiers participating in the Philippines-US Exercise Balikatan in 2014[17].

In this study, the conventional culture-dependent method and a commercially available milk bacterial DNA kit were used to determine the occurrence of *C. coli* and *C. jejuni* in the raw milk of backyard-raised carabaos in an agricultural area in the Philippines. The gene primer for *lpxA* gene was chosen to confirm the identity of the isolate because of its ability to distinguish among *Campylobacter* spp.[18]. In addition, the presence of phenotypic and genotypic resistance to common antibiotics was also determined. The 16S gene sequencing was used to identify other microorganisms that grew on the *Campylobacter* selective media and competed with the growth of the target microorganism. This information will help in determining the future course of action in the monitoring of *Campylobacter* spp., in milk in the Philippines.

MATERIALS AND METHODS

Study area: Raw milk samples were collected from backyard carabao farms in the Province of Nueva Ecija, Philippines from June to November, 2023. Milk samples were taken between the wet months of June to August and the dry months of September to November.

Milk sample collection and processing: One hundred seven milk samples were collected from the study area. Around 200 mL of the unpasteurized milk samples were taken from the milk bucket/milk bottle, placed on sterilized brown bottles and transported on ice to the laboratory for analysis. Before processing, the pH of the milk samples was checked and maintained within pH 7.5±0.2. All milk samples were processed within the day of collection.

***Campylobacter* detection using the conventional method:** Analysis of milk samples followed the procedure outlined in Bacteriological Analytical Manual with modifications[19]. Twenty milliliters of the raw milk samples were centrifuged at 14000 rpm for 20 min to separate the milk pellets from the fat layer and supernatant. For the enrichment step, the fat layer and supernatant were discarded and milk pellets were placed in 40 mL Bolton broth supplemented with 5% lyzed horse blood and Bolton broth antibiotic supplements (cefoperazone 10 mg, vancomycin 10 mg, trimethoprim 10 mg and amphotericin B 5 mg). The mixture is then incubated at 42°C for 48 hrs under microaerobic conditions.

After incubation, a loopful of the broth was streaked onto Modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA) supplemented with antibiotics (cefoperazone, 16 mg; amphotericin B, 5 mg) and the plates were incubated at 42°C for 48 to 72 hrs under microaerobic condition. Positive controls spiked with ATCC strain of *C. coli* (ATCC®43478) and *C. jejuni* (ATCC®33560) and negative controls using Ultra High Temperature (UHT) milk and Bolton broth with 5% lyzed horse blood but without milk were included in the analysis of every batch of samples to check for contamination. All samples were analyzed in duplicates.

Colonies resembling *Campylobacter* colonies were picked from the mCCDA plates and streaked onto a series of mCCDA plates to facilitate isolation of a pure culture of *Campylobacter*. Suspected colonies of *Campylobacter* were further streaked onto Columbia blood agar supplemented with 5% defibrinated sheep blood and incubated accordingly. Colony characteristics, gram staining reaction and biochemical tests including catalase test, Hippurate test, reaction in Triple Sugar Iron (TSI) and LIA were used for phenotypic identification of presumptive *Campylobacter* colonies. The DNA of the presumptive *Campylobacters* are extracted using heat lysis and confirmed using PCR. The DNA extracts are then amplified in the PCR and checked with gel electrophoresis or stored in the -20°C freezer for future analysis.

***Campylobacter* detection using the commercial milk bacterial DNA kit:** A commercial milk bacterial DNA isolation kit (Norgen Biotek, Corp) was used to determine the presence of *Campylobacter* DNA in the raw milk samples and provide a comparison with the culture-dependent method. The use of the commercial kit followed the manufacturer's recommendation. Briefly, replicate samples of 1 mL of raw milk were placed into a microcentrifuge tube and centrifuged at 14000 rpm for 3 min. The supernatant and fat layer were removed leaving the milk pellet in the tube. The milk pellet is then re-suspended in a buffer and mixed well using a vortex mixer to lyse the bacterial cells. Ethanol was added to the lysate and the solution was loaded onto a spin column. Afterwards, the column was washed and the purified bacterial DNA was amplified in the PCR for subsequent analysis or stored at -20°C for future analysis.

Genotypic analysis of *Campylobacter* and its competing microflora using PCR: The DNA extracts from presumptive *Campylobacter* colonies and the commercial kit were confirmed by PCR using primers that encode for *lpxA* gene of *C. jejuni* and *C. coli*. forward primers that encode for lpxA nucleotide sequence of *C. jejuni* (*lpxA C. jejuni*) and *C. coli* (*lpxA C. coli*) were used with the reverse primer, *lpxARKK2m*. The primers used in the study were shown in Table 1. Initial denaturation was set at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 50°C for 30 sec, elongation at 72°C for 1 min and lastly 5 min of final extension at 72°C. Samples showing amplicon sizes of 391 and 331 bp were considered positive for *C. coli* and *C. jejuni*, respectively.

The DNA of the bacterial colonies that grew on mCCDA but were determined to be negative for *C. coli* and *C. jejuni* in the PCR were extracted via heat lysis and sequenced using 16S rRNA to determine the identity of the microorganisms. The DNA amplicons were taken to the Philippine Carabao Center, Science City of Munoz, Nueva Ecija, Philippines for gene sequencing and the sequences were compared to those in gene bank and confirmed using BLAST software.

Analysis of Antimicrobial Resistance (AMR) in the *Campylobacter* isolates

Antibiotic susceptibility testing using disk diffusion assay: Colonies from cultures that were confirmed to be *Campylobacter* by PCR were processed and analyzed for susceptibility or resistance to several antibiotics. A bacterial suspension was prepared and standardized using 0.5 McFarland standard. A swab was taken from the suspension and streaked onto the Mueller-Hinton Agar (MHA) with 5% defibrinated horse blood. To ensure a uniform layer of bacteria, the inoculum was spread onto the surface of the agar while the plates were rotated twice. Afterwards, the antibiotic discs (ciprofloxacin, tetracycline 30 µg, ampicillin 5 µg) were placed on the surface of the inoculated MHA using a pair of sterilized forceps. Light pressure was applied on the discs to ensure that the discs were completely touching the surface of the agar. The plates were incubated in an inverted position at 42°C in a microaerophilic environment for 18 to 24 hrs. After incubation, the diameter of the zone of inhibition (IZD) was measured using a digital caliper. The CLSI Guidelines were used as a basis to determine the susceptibility of the *Campylobacter* isolate[20].

Table 1: List of primers used in this study

Target bacteria	Gene	Primer sequence 5'-3'	Amplicon size (bp)	References
Campylobacter jejuni	*lpxA*	F'-ACA ACT TGG TGA CGA TGT TGT A	331	Klena *et al.*[18]
	lpxARKK2m	R-CAA TCA TGD GCD ATA TGA SAA TAH GCC AT		
Campylobacter coli	*lpxA*	F-AGA CAA ATA AGA GAG AAT CAG	391	Klena *et al.*[18]
	lpxARKK2m	R-CAA TCA TGD GCD ATA TGA SAA TAH GCC AT		
Bacteria	*16S rRNA*	R- ACG GCT ACC TTG TTA CGA CTT		
Ampicillin resistance	*blaOXA-61*	F- AGA GTA TAA TAC AAG CG		
		R-TAG TGA GTT GTC AAG CC	372	Gibreel *et al.*[22]
Tetracycline resistance	*tet(O)*	F- GGC GTT TTG TTT ATG TGC G		
		R- ATG GAC AAC CCG ACA GAA GC	559	Christidis *et al.*[23]

Molecular analysis of antimicrobial resistance genes: The PCR-detected *C. coli* isolate was subjected for the detection of genes encoding for tetracycline and ampicillin resistance targeting *tetO*[21] and *blaOXA-61*[22], respectively (Table 1). The amplification condition included initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 56°C for 45 sec, extension at 72°C for 1 min and lastly 10 min of final extension at 72°C. The DNA products were then subjected to gel electrophoresis on a 1.0% agarose gel run at 100 V for 20 min and stained with GelRed® Nucleic Acid Gel Stain (Biotium, USA).

Samples that exhibited amplicon sizes of 559 and 372 bp were considered positive for tetracycline and ampicillin resistance genes, respectively.

RESULTS

Frequency of *C. coli* contamination in raw carabaos milk: Primers that encode for the *lpxA* gene were used to detect the presence of *C. coli* and *C. jejuni* from 107 raw milk samples collected from backyard-raised carabaos in the Province of Nueva Ecija, Philippines. Several presumptive colonies based on phenotypic characteristics were isolated from the selective media but only one sample (S16) was confirmed as *C. coli* by PCR (Fig. 1). On the other hand, analysis using the commercial milk bacterial DNA kit revealed that seven milk samples or 6.54% contained the lipid A gene of *C. coli* (Fig. 2), but no *C. jejuni* was detected in any of the samples. It must be noted that only one sample (S16) was found positive for *C. coli* using both methods.

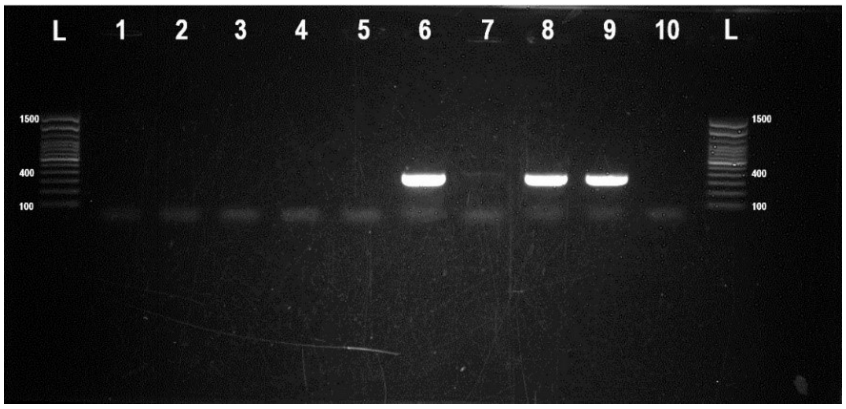

Fig. 1: Gel electrophoresis image of PCR-confirmed *C. coli* (lane 6 and 8; 391 bp), positive control (lane 9) using *C. coli* ATCC®43478 in 1% agarose gel, lane L (DNA ladder, 100 bp)
Primers used are *lpxA* (forward) and *lpxA-RKK2m* (reverse)

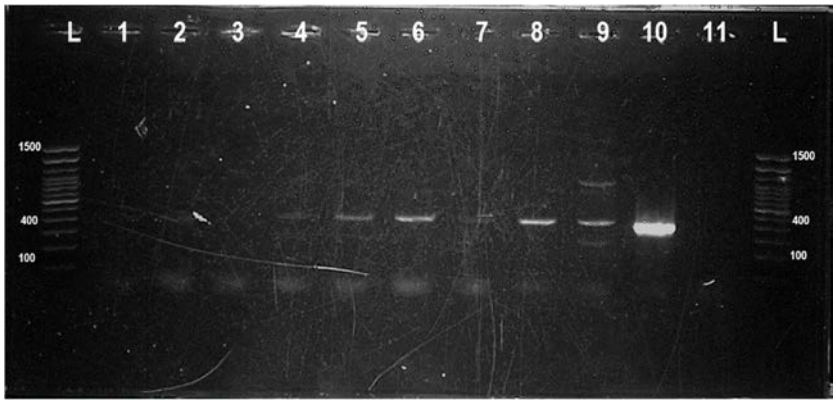

Fig. 2: Gel electrophoresis image of PCR products extracted using milk bacterial DNA Kit with primers *lpxA* (forward) and *lpxA-RKK2m* (reverse) for *C. coli* (391 bp)
Lanes 5, 6, 8, 9 are *C. coli* positive samples, Lane 10 is positive control using *C. coli* ATCC®43478

Table 2: Result of disk diffusion assay on *Campylobacter* isolates

Parameter	Ciprofloxacin (5 µg)	Tetracycline (30 µg)	Ampicillin (10 µg)
Average IZD	50.18	33.75	13.90
***Zone diameter interpretative criteria**			
Sensitive	≥24	≥26	≥17
Intermediate	21-23	23-25	14-16
Resistant	≤20	≤22	≤13

IZD: Diameter of the zone of inhibition (mm) and *Comparison with the result

Phenotypic and genotypic detection of AMR: Antibiotic susceptibility testing of the isolate using disks that contains ampicillin, ciprofloxacin and tetracycline revealed, that the isolate is susceptible to ciprofloxacin (IZD = 50.18 mm) and tetracycline (IZD = 33.75 mm) based on CLSI Guidelines result showed intermediate resistance to ampicillin (IZD = 13.90 mm) (Table 2). On the other hand, molecular methods did not detect the presence of any genes encoding for tetracycline and ampicillin resistance.

Competing microflora of *Campylobacter*: Several other bacterial colonies were found to grow on mCCDA and these were observed to interfere with the growth of the more fastidious and slow-growing *Campylobacters*. Gram-staining and PCR analysis showed these are not *Campylobacters*. The 16S gene sequencing of the colonies revealed that out of the 22 colonies that were sequenced, majority are *Acinetobacter baumannii* (59.00%), followed by *Pseudomonas aeruginosa* (23.00%). *Pseudoroseomonas cervicalis*, *Klebsiella pneumoniae*, *Roseomonas cervicalis* and *Acinetobacter pittii* were also detected from the selective agars used to isolate *Campylobacter* spp. (Appendix A: Supplemental data).

DISCUSSION

This study established the presence of *C. coli* in raw carabaos' milk using both the conventional culture method and the milk bacterial DNA kit with primer for lipid A gene, *lpxA.* The presence of *C. coli* in the raw milk samples in this study is relatively low (0.94% for the culture method and 6.54% using molecular technique) but did not widely diverge from the results of other studies. A meta-analysis of *Campylobacter* levels in raw milk from the US, Europe and New Zealand showed a prevalence rate of 1.64%[23] while a global prevalence rate of *Campylobacter* spp., was recorded at 4.00%[24]. It is also established that the prevalence of *Campylobacter* spp., in milk is lower than those recorded in other foods of animal origin such as chicken[25] and red meat[26]. After chicken, however, milk is the second most common source of *Campylobacter* infection[27].

A difference in the detection rates between the conventional culture method and the use of milk bacterial DNA kit seen in this study, i.e., higher detection rates when the milk bacterial DNA kit was used, is in line with the results of other studies that compared the conventional culture-dependent with molecular methods[28,29]. The discrepancy was attributed to several factors including the difficult isolation of the fastidious *Campylobacter* spp. and the presence of Viable But Non-Culturable (VBNC) *Campylobacter*.

The discrepancy between the result of molecular detection and the culture method which has only successfully isolated *C. coli* from one sample, pointed to the possible presence of the VBNC form in the milk samples. *Campylobacters* are known to enter this state to survive various stress conditions including low temperature, presence of oxygen and starvation, however, the microorganisms were undetectable using the conventional culture method[30]. It must be noted, however, that even though VBNC microorganisms are dormant they can still infect once resuscitated[31] making VBNC a significant threat to public health. This is true for *Campylobacter* spp., which are known to enter and stay in this state for several months and be resuscitated in pathogen-free fertilized egg[32] or mouse' intestine[33].

The difficult isolation of *Campylobacter* is due in part to the presence of other milk constituents such as protein, milk fats and the natural milk microbiota[34]. Other microorganisms in milk also have the tendency to grow faster overwhelming the growth of the more fastidious *Campylobacter*. *Acinetobacter baumannii* and *Pseudomonas aeruginosa*, the major competing microorganisms detected in this study have also been reported by studies of water samples collected from waste stabilization pond[35] and in fresh produce like sprouts and lettuce[36]. Although normally found in the soil, *A. baumannii* are opportunistic pathogens and are well-known to possess plasmids that can serve as platforms for AMR dissemination[37]. Its presence in raw milk is a cause for concern because *Acinetobacters* are known to possess innate resistance to most antibiotics[38] and because it is known that resistance genes can be transferred horizontally from one bacterium to another bacterium[39]. *Acinetobacter baumannii* therefore, tends to become sources of the resistance genes for other microorganisms[40,41].

The increasing number of studies reported the presence of genes in *Campylobacter* spp., that afford resistance against commonly used antibiotics including fluoroquinolone, tetracycline, ampicillin, erythromycin and gentamicin[42]. While the development of antibiotic resistance in microorganisms is said to be partly due to the misuse of antibiotics, there are reports on the detection of antimicrobial resistance in *Campylobacter* from food even in the absence of antibiotic use[43]. This suggests that other mechanisms, such as horizontal gene transfer can possibly cause the acquisition of resistance genes in microorganisms. It must be noted that milk has high nutritional content that allows the growth of several microorganisms, transfer of genes among these microorganisms is possible.

Whole genome sequencing and *in vitro* antimicrobial susceptibility testing of *Campylobacter* spp., showed 100% correlation between its genotypic and phenotypic resistance[44]. This was not observed in the current study as no resistance to ampicillin is detected by PCR, but instead, phenotypic tests showed intermediate resistance to ampicillin. Moderate discrepancies between the result of genotypic and phenotypic testing for AMR, have been observed in other microorganisms, including *E. coli*[45], *K. pneumoniae*[46] and non-tyhoidal *S. enterica*[47]. Even though low in occurrence, understanding the reasons for the discrepancy is still important due to the possible consequences in clinical medicine and for the proper surveillance of AMR.

The prevalence of *Campylobacter* in a variety of samples appeared to be affected by several factors including landscape and seasons. Studies revealed that the prevalence is higher in sites with more agricultural land use and poultry production and is correlated with streamflow, temperature and dissolved oxygen[48]. On the other hand, seasonal influence is variable with some studies showing higher *Campylobacter* prevalence during hot season[49] while some studies reported higher prevalence rates during rainy season[50]. In this study, it is observed that all of the samples that carry *C. coli* DNA were collected during rainy periods suggesting that rain splashes can be an important route of contamination. Further investigation is recommended to identify other possible sources of contamination including the analysis of soil, water and milk collection equipment.

CONCLUSION

To the best of the authors' knowledge, this study is the first to investigate the presence of *Campylobacters* in the raw milk of carabaos in the Philippines. Results revealed the presence of *C. coli*, but no *C. jejuni* was detected. This study also demonstrated the presence phenotypic resistance against ampicillin suggesting that raw carabaos' milk has the potential to cause public health problems. In addition, the detection of *C. coli* in samples collected during the rainy season, suggests that further studies on the seasonal prevalence of *Campylobacter* spp., should be conducted as this can help in determining possible routes of milk contamination.

SIGNIFICANCE STATEMENT

Carabaos' milk can be an inexpensive and locally available source of good protein for the rural Philippines, ensuring that it is safe for consumption should be a priority. To the best of the authors' knowledge, this is the first study in the country to investigate the presence and anti-microbial resistance of *C. coli* in the raw milk of carabaos. Using molecular methods, 6.54% of the 107 milk samples tested positive for the presence of *Campylobacter coli* genes; while antibiotic disk susceptibility assay showed that the isolates have intermediate resistance to ampicillin. Results suggest a review of the existing protocol in handling raw carabao's milk is necessary to prevent risk to public health safety.

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REFERENCES

1. Davis, C.G., J.G. Cessna and D.P. Blayney, 2018. Southeast Asia's import demand for skim milk powder: Implications for US exporters. J. Dairy Sci., 101: 4676-4689.
2. Wanjala, W.N., J.M. Nduko and M.C. Mwende, 2018. Coliforms contamination and hygienic status of milk chain in emerging economies. J. Food Qual. Hazards Control, 5: 3-10.
3. Leedom, J.M., 2006. Clinical practice: Milk of nonhuman origin and infectious diseases in humans. Clin. Infect. Dis., 43: 610-615.

4. Ntuli, V., T. Sibanda, J.A. Elegbeleye, D.T. Mugadza, E. Seifu and E.M. Buys, 2023. Dairy Production: Microbial Safety of Raw Milk and Processed Milk Products. In: Present Knowledge in Food Safety: A Risk-Based Approach Through the Food Chain, Knowles, M.E., L.E. Anelich, A.R. Boobis and B. Popping (Eds.), Academic Press, United States, ISBN: 9780128194706, pp: 439-454.
5. Kaakoush, N.O., N. Castaño-Rodríguez, H.M. Mitchell and S.M. Man, 2015. Global epidemiology of *Campylobacter* infection. Clin. Microbiol. Rev., 28: 687-720.
6. McCarthy, N. and J. Giesecke, 2001. Incidence of Guillain-Barré Syndrome following infection with *Campylobacter jejuni*. Am. J. Epidemiol., 153: 610-614.
7. Keithlin, J., J. Sargeant, M.K. Thomas and A. Fazil, 2014. Systematic review and meta-analysis of the proportion of *Campylobacter cases* that develop chronic sequelae. BMC Public Health, Vol. 14. 10.1186/1471-2458-14-1203.
8. Pope, J.E., A. Krizova, A.X. Garg, H. Thiessen-Philbrook and J.M. Ouimet, 2007. Campylobacter reactive arthritis: A systematic review. Sem. Arthritis Rheumatism, 37: 48-55.
9. Devleesschauwer, B., M. Bouwknegt, M.J.J. Mangen and A.H. Havelaar, 2017. Health and Economic Burden of *Campylobacter*. In: Campylobacter: Features, Detection, and Prevention of Foodborne Disease, Klein, G. (Ed.), Academic Press, Cambridge, Massachusetts, ISBN: 9780128036235, pp: 27-40.
10. Plishka, M., J.M. Sargeant, A.L. Greer, S. Hookey and C. Winder, 2021. The prevalence of *Campylobacter* in live cattle, Turkey, chicken, and swine in the United States and Canada: A systematic review and meta-analysis. Foodborne Pathog. Dis., 18: 230-242.
11. Al Hakeem, W.G., S. Fathima, R. Shanmugasundaram and R.K. Selvaraj, 2022. *Campylobacter jejuni* in poultry: Pathogenesis and control strategies. Microorganisms, Vol. 10. 10.3390/microorganisms10112134.
12. Lim, P.W.N., D.C. Tiam-Lee, P.A.P. Paclibare, M.S.E.P. Subejano, J.A.S. Cabero-Palma and G.M. Penuliar, 2017. High rates of contamination of poultry meat products with drug-resistant *Campylobacter* in Metro Manila, Philippines. Jpn. J. Infect. Dis., 70: 311-313.
13. Sison, F.B., W. Chaisowwong, T. Alter, S. Tiwananthagorn and D. Pichpol *et al*., 2014. Loads and antimicrobial resistance of *Campylobacter* spp. on fresh chicken meat in Nueva Ecija, Philippines. Poult. Sci., 93: 1270-1273.
14. Barrios, P.R., J. Reiersen, R. Lowman, J.R. Bisaillon and P. Michel *et al*., 2006. Risk factors for *Campylobacter* spp. colonization in broiler flocks in Iceland. Preventive Vet. Med., 74: 264-278.
15. Hatta, Y., T. Omatsu, S. Tsuchiaka, Y. Katayama and S. Taniguchi *et al*., 2016. Detection of *Campylobacter jejuni* in rectal swab samples from *Rousettus amplexicaudatus* in the Philippines. J. Vet. Med. Sci., 78: 1347-1350.
16. Adkins, H.J., J. Escamilla, L.T. Santiago, C. Rañoa, P. Echeverria and J.H. Cross, 1987. Two-year survey of etiologic agents of diarrheal disease at San Lazaro Hospital, Manila, Republic of the Philippines. J. Clin. Microbiol., 25: 1143-1147.
17. Lertsethtakarn, P., K. Nakjarung, S. Silapong, P. Neesanant and P. Sakpaisal *et al*., 2016. Detection of diarrhea etiology among U.S. military personnel during exercise Balikatan 2014, Philippines, using taqman array cards. Mil. Med., 181: e1669-e1674.
18. Klena, J.D., C.T. Parker, K. Knibb, J.C. Ibbitt and P.M.L. Devane *et al*., 2004. Differentiation of *Campylobacter coli*, *Campylobacter jejuni*, *Campylobacter lari*, and *Campylobacter upsaliensis* by a multiplex PCR developed from the nucleotide sequence of the lipid a gene *lpxA*. J. Clin. Microbiol., 42: 5549-5557.
19. Modi, S., M.N. Brahmbhatt, Y.A. Chatur and J.B. Nayak, 2015. Prevalence of *Campylobacter* species in milk and milk products, their virulence gene profile and antibiogram. Vet. World, 8: 1-8.
20. Humphries, R.M., J. Ambler, S.L. Mitchell, M. Castanheira and T. Dingle *et al*., 2018. CLSI methods development and standardization working group best practices for evaluation of antimicrobial susceptibility tests. J. Clin. Microbiol., Vol. 56. 10.1128/JCM.01934-17.
21. Obeng, A.S., H. Rickard, M. Sexton, Y. Pang, H. Peng and M. Barton, 2012. Antimicrobial susceptibilities and resistance genes in *Campylobacter* strains isolated from poultry and pigs in Australia. J. Appl. Microbiol., 113: 294-307.
22. Gibreel, A., D.M. Tracz, L. Nonaka, T.M. Ngo, S.R. Connell and D.E. Taylor, 2004. Incidence of antibiotic resistance in *Campylobacter jejuni* isolated in Alberta, Canada, from 1999 to 2002, with special reference to *tet*(O)-mediated tetracycline resistance. Antimicrob. Agents Chemother., 48: 3442-3450.
23. Christidis, T., K.D.M. Pintar, A.J. Butler, A. Nesbitt, M.K. Thomas, B. Marshall and F. Pollari, 2016. *Campylobacter* spp. prevalence and levels in raw milk: A systematic review and meta-analysis. J. Food Prot., 79: 1775-1783.
24. Taghizadeh, M., A. Nematollahi, M. Bashiry, F. Javanmardi, M. Mousavi and H. Hosseini, 2022. The global prevalence of *Campylobacter* spp. in milk: A systematic review and meta-analysis. Int. Dairy J., Vol. 133. 10.1016/j.idairyj.2022.105423.
25. Padungtod, P. and J.B. Kaneene, 2005. *Campylobacter* in food animals and humans in Northern Thailand. J. Food Prot., 68: 2519-2526.
26. Raeisi, M., R. Khoshbakht, E.A. Ghaemi, M. Bayani, M. Hashemi, N.S. Seyedghasemi and H. Shirzad-Aski, 2017. Antimicrobial resistance and virulence-associated genes of *Campylobacter* spp. isolated from raw milk, fish, poultry, and red meat. Microb. Drug Resist., 23: 925-933.

27. Hussain, I., M.S. Mahmood, M. Akhtar and A. Khan, 2007. Prevalence of *Campylobacter* species in meat, milk and other food commodities in Pakistan. Food Microbiol., 24: 219-222.
28. Šabatková, Z., K. Demnerová and J. Pazlarová, 2008. Optimisation of the PCR method for the detection of *Campylobacter jejuni* and *Campylobacter coli* in samples of ready-to-eat chicken meals. Czech J. Food Sci., 26: 291-297.
29. Khanzadi, S., A. Jamshidi, V. Soltaninejad and S. Khajenasiri, 2010. Isolation and identification of *Campylobacter jejuni* from bulk tank milk in Mashhad-Iran. World Appl. Sci. J., 9: 638-643.
30. Lv, R., K. Wang, J. Feng, D.D. Heeney, D. Liu and X. Lu, 2019. Detection and quantification of viable but non-culturable *Campylobacter jejuni*. Front. Microbiol., Vol. 10. 10.3389/fmicb.2019.02920.
31. Li, L., N. Mendis, H. Trigui, J.D. Oliver and S.P. Faucher, 2014. The importance of the viable but non-culturable state in human bacterial pathogens. Front. Microbiol., Vol. 5. 10.3389/fmicb.2014.00258.
32. Chaveerach, P., A.A.H.M. ter Huurne, L.J.A. Lipman and F. van Knapen, 2003. Survival and resuscitation of ten strains of *Campylobacter jejuni* and *Campylobacter coli* under acid conditions. Appl. Environ. Microbiol., 69: 711-714.
33. Baffone, W., A. Casaroli, B. Citterio, L. Pierfelici and R. Campana *et al*., 2006. *Campylobacter jejuni* loss of culturability in aqueous microcosms and ability to resuscitate in a mouse model. Int. J. Food Microbiol., 107: 83-91.
34. Baylis, C.L., S. MacPhee, K.W. Martin, T.J. Humphrey and R.P. Betts, 2000. Comparison of three enrichment media for the isolation of *Campylobacter* spp. from foods. J. Appl. Microbiol., 89: 884-891.
35. Sheludchenko, M., A. Padovan, M. Katouli and H. Stratton, 2020. *Acinetobacter baumannii* detected on modified charcoal-cefoperazone-deoxycholate agar in a waste stabilization pond. Can. J. Microbiol., 66: 206-213.
36. Chon, J.W., J.Y. Jung, Y. Ahn, D. Bae and S. Khan *et al*., 2021. Detection of *Campylobacter jejuni* from fresh produce: Comparison of culture- and PCR-based techniques, and metagenomic approach for analyses of the microbiome before and after enrichment. J. Food Prot., 84: 1704-1712.
37. Cameranesi, M.M., J. Morán-Barrio, A.S. Limansky, G.D. Repizo and A.M. Viale, 2018. Site-specific recombination at XerC/D sites mediates the formation and resolution of plasmid co-integrates carrying a blaOXA-58- and TnaphA6-resistance module in *Acinetobacter baumannii*. Front. Microbiol., Vol. 9. 10.3389/fmicb.2018.00066.
38. Sarshar, M., P. Behzadi, D. Scribano, A.T. Palamara and C. Ambrosi, 2021. *Acinetobacter baumannii*: An ancient commensal with weapons of a pathogen. Pathogens, Vol. 10. 10.3390/pathogens10040387.
39. da Silva, G.J. and S. Domingues, 2016. Insights on the horizontal gene transfer of carbapenemase determinants in the opportunistic pathogen *Acinetobacter baumannii*. Microorganisms, Vol. 4. 10.3390/microorganisms4030029.
40. Maeusli, M., B. Lee, S. Miller, Z. Reyna and P. Lu *et al*., 2020. Horizontal gene transfer of antibiotic resistance from *Acinetobacter baylyi* to *Escherichia coli* on lettuce and subsequent antibiotic resistance transmission to the gut microbiome. mSphere, Vol. 5. 10.1128/mSphere.00329-20.
41. Leungtongkam, U., R. Thummeepak, K. Tasanapak and S. Sitthisak, 2018. Acquisition and transfer of antibiotic resistance genes in association with conjugative plasmid or class 1 integrons of *Acinetobacter baumannii*. PLoS ONE, Vol. 13. 10.1371/journal.pone.0208468.
42. Qin, X., X. Wang and Z. Shen, 2023. The rise of antibiotic resistance in *Campylobacter*. Curr. Opin. Gastroenterol., 39: 9-15.
43. Abraham, S., S. Sahibzada, K. Hewson, T. Laird and R. Abraham *et al*., 2020. Emergence of fluoroquinolone-resistant *Campylobacter jejuni* and *Campylobacter coli* among Australian chickens in the absence of fluoroquinolone use. Appl. Environ. Microbiol., Vol. 86. 10.1128/AEM.02765-19.
44. Zhao, S., G.H. Tyson, Y. Chen, C. Li and S. Mukherjee *et al*., 2016. Whole-genome sequencing analysis accurately predicts antimicrobial resistance phenotypes in *Campylobacter* spp. Appl. Environ. Microbiol., 82: 459-466.
45. Duy, D.T., N.L.L. Anh, N.T.K. Thoa, K. Phitsanuwattana and S. Thongratsakul *et al*., 2021. Identification of genotype and phenotype of antimicrobial resistance of *Escherichia coli* isolates from pigs in Southern Vietnam. Thai J. Vet. Med., 51: 125-132.
46. Urmi, U.L., S. Nahar, M. Rana, F. Sultana and N. Jahan *et al*., 2020. Genotypic to phenotypic resistance discrepancies identified involving β-lactamase genes, *bla*KPC, *bla*IMP, *bla*NDM-1, and *bla*VIM in uropathogenic *Klebsiella pneumoniae*. Infect. Drug Resist., 13: 2863-2875.
47. Schwan, C.L., S. Lomonaco, L.M. Bastos, P.W. Cook and J. Maher *et al*., 2021. Genotypic and phenotypic characterization of antimicrobial resistance profiles in non-typhoidal *Salmonella enterica* strains isolated from Cambodian informal markets. Front. Microbiol., Vol. 12. 10.3389/fmicb.2021.711472.
48. Vereen Jr., E., R.R. Lowrance, M.B. Jenkins, P. Adams, S. Rajeev and E.K. Lipp, 2013. Landscape and seasonal factors influence *Salmonella* and *Campylobacter* prevalence in a rural mixed use watershed. Water Res., 47: 6075-6085.
49. Omara, S.T., H.A. El Fadaly and A.M.A. Barakat, 2015. Public health hazard of zoonotic *Campylobacter jejuni* reference to Egyptian regional and seasonal variations. Res. J. Microbiol., 10: 343-354.
50. Wanja, D.W., P.G. Mbuthia, G.O. Aboge and L.C. Bebora, 2022. Seasonal prevalence and molecular identification of thermophilic *Campylobacter* from chicken, cattle, and respective drinking water in Kajiado County, Kenya. Int. J. Microbiol., Vol. 2022. 10.1155/2022/1526641.

APPENDIX A

Appendix A: Supplemental data on the result of gene sequencing of competing microflora

Sample ID	Bacteria	Query cover	Percent identity	length (bp)
S7	*Pseudoroseomonas cervicalis*	99	98.41	726
	Roseomonas cervicalis	85	97.85	996
S12	*Pseudomonas aeruginosa*	99	99.76	1102
	Pseudomonas aeruginosa	100	98.27	989
	Pseudomonas aeruginosa	100	99.5	940
S13	*Acinetobacter baumannii*	67	99.66	865
S15	*Acinetobacter pittii*	86	99.64	833
S16	*Acinetobacter baumannii*	100	100	958
	Acinetobacter baumannii	100	99.14	839
	Acinetobacter baumannii	99	99.31	1027
	Acinetobacter baumannii	100	99.2	812
	Klebsiella pneumoniae	93	99.83	1072
	Acinetobacter baumannii	100	99.97	894
M5	*Pseudomonas aeruginosa*	100	100	863
M7	*Acinetobacter baumannii*	100	99.09	1019
M10	*Pseudomonas aeruginosa*	100	99.79	848
M11	*Acinetobacter baumannii*	100	100	858
M46	*Acinetobacter baumannii*	79	97.89	939
M49	*Acinetobacter baumanii*	100	98.78	1003
M52	*Acinetobacter baumanii*	91	99.76	860
M56	*Acinteobacter baumanii*	99	100	992
M57	*Acinetobacter baumanii*	93	99.53	689